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THE SEPARATION OF PGB INTO MOLECULAR WEIGHT CLASSES BY DIALYSIS--ETC(U)
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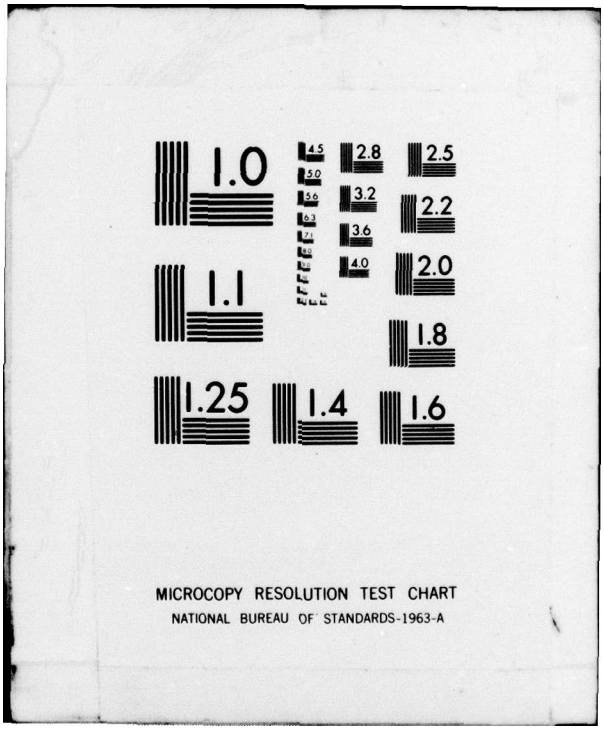
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LEVEL II

THE SEPARATION OF PGB_x INTO MOLECULAR WEIGHT CLASSES BY DIALYSIS

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PGB_x was separated into two molecular weight groups by dialysis against phosphate buffer between pH 6.5-7.8. The dialysate contained from 9.3 percent to 38.2 percent of the total PGB_x dialysed. When PGB_x was dialysed against methanol, 90.5 percent passed through the dialysis membrane.

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INTRODUCTION

The importance of PGB_x* in the reversal of the effects of myocardial ischemia and cerebral ischemia in animals has been documented (1, 2) and has been confirmed in other laboratories (3, 4). Because of these unique properties, the use of PGB_x in the treatment of similar type human diseases appears reasonable. However, the heterogeneity of the PGB_x preparations synthesized according to Polis et al (5) introduces a possible obstacle for its use with humans. In order to overcome this difficulty, studies have been undertaken at the Naval Air Development Center to obtain highly purified preparations of PGB_x so that interferences by/or side-effects from contaminants would be minimized.

In preliminary experiments, Polis (6) studied the use of solvent extraction, adsorption chromatography, ion-exchange chromatography, thin layer chromatography, reverse phase chromatography, etc. in order to purify PGB_x. Of all the methods studied, only molecular exclusion chromatography (MEC) on Sephadex LH-20 (Pharmacia, Inc., Piscataway, NJ) appeared to afford any degree of purification. This methodology was then incorporated into the general preparation of PGB_x (5). Even after this type of chromatography, the PGB_x obtained was still a highly complex mixture. More recently in this laboratory it was found that PGB_x could be separated into two molecular size classes by aqueous gel filtration on Sephadex G-100 or G-150 (7). This separation, based on molecular sizing, suggested dialysis as a possible additional method for the fractionation of PGB_x. This report describes the separation of PGB_x into molecular weight classes by ordinary dialysis.

EXPERIMENTAL

PGB_x was prepared according to the method described by Polis et al (5). Dialysis was carried out with commercially available cellophane tubing which had a molecular weight cut-off of 12000. Approximately 100 mg of PGB_x preparation no. 27 were dissolved in the dialysis solvent at a concentration of 10-50 mg per ml, placed inside the dialysis tubing and then immersed in 250 ml of solvent. During this period, the external solution was stirred so as to attain dialysis equilibrium rapidly. After 24 hours the dialysate was replaced with fresh solvent and the dialysis continued for an additional 24 hours. The PGB_x remaining inside the dialysis tubing was removed quantitatively. The two dialysates were combined and concentrated as described below. When methanol was used as the dialysis solvent, the solvent was removed by flash evaporation at 45° under reduced pressure. When aqueous phosphate buffer was used, the combined dialysates were acidified to pH 3.0 with perchloric acid and then shaken with isobutanol. The isobutanol layer was separated and washed with water to remove excess acid. The isobutanol solvent was flash evaporated off at 45° under reduced pressure. The retentates were concentrated in a similar manner. The PGB_x residue was dissolved

* Small amounts of PGB_x are available from the authors upon application from qualified investigators.

in ethanol and stored at 4° until used. UV spectra were measured in ethanol using a Cary Model 14 recording spectrophotometer (Sunnyvale, CA). Molecular weights were measured by vapor pressure osmometry of free acids dissolved in methanol, using a Wescan apparatus (Wescan Instruments, Inc., Santa Clara, CA). The assay of PGB_x on the *in vitro* phosphorylation of degraded isolated mitochondria was performed by the method of Polis *et al* (5). For purposes of quantification, a unit of PGB_x activity was defined as the inverse ratio of the amount of PGB_x (in μg) required to restore 50 percent of the phosphorylation (3.0 μmoles esterified phosphate) to that amount required with the standard PGB_x preparation. For this purpose the best fitting curve of the rising portion of the PGB_x concentration vs activity curve was calculated by the method of linear regression to yield the value of constants, a_o and a_1, in the equation that describes the curve: $Y = a_o + a_1X$. By substituting 3 for Y and solving for X, the amount of PGB_x required for 50 percent recovery of activity was obtained. The unit of PGB_x activity was then defined as

$$K_a = \frac{\text{X } PGB_x \text{ standard*}}{\text{X } PGB_x \text{ unknown}} .$$

The K_a values calculated in this manner describe the activity of the various PGB_x preparations as follows:

When $K_a = 1$, activity of unknown = activity of standard

$K_a < 1$, activity of unknown less than standard

$K_a > 1$, activity of unknown more than standard.

In addition, if the total amount of PGB_x (in mg) is multiplied by the K_a value the numerical figure obtained is essentially a measure of the total activity of the sample. Thus the recovery of the activity may be followed during any fractionation procedure.

RESULTS AND DISCUSSION

Table I lists the distribution of fraction weight, *in vitro* activity and molecular weight of PGB_x recovered after dialysis against the different solvents used in this study. In the first dialysis experiment, 109.6 mg of PGB_x combination 2A was dialysed against 100 percent methanol at 3°. The material remaining inside the dialysis tubing was only 10 percent of the total weight and 7.5 percent of the total *in vitro* PGB_x activity. The amount recovered was too low for molecular weight measurement. The dialysate containing 90.5 percent of the total PGB_x gave a value of 1510 for the molecular weight, implying that the retentate should be higher than the starting molecular weight (2077).

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* A program for use with the Hewlett Packard Model 97 programable calculator is available from the author for the calculation of K_a.

When PGB_x was dialysed against 0.05 M phosphate buffer pH 7.8, 66 percent of the total weight remained inside the dialysis tubing with 75 percent of the total *in vitro* activity. The molecular weight (MW) of this fraction rose to 2804. The pH 7.8 dialysate contained 34 percent of the original weight and 25 percent of the *in vitro* activity with a drop in the MW to 1849. The PGB_x fractions recovered following pH 7.2 dialysis showed 62 percent of the total weight and 53 percent of the *in vitro* activity non-dialysable. This fraction had a MW of 2576, approximately that of the PGB_x before dialysis. The dialyzable fraction of PGB_x amounted to 38 percent with 47 percent of the *in vitro* activity and exhibited a MW of 1860. At pH 6.5, 91 percent of PGB_x with 91 percent of the *in vitro* activity was non-dialyzable. This fraction yielded a MW of 2694. The dialysate containing 9.3 percent of the weight with only 9 percent of the *in vitro* activity was too small a quantity for MW measurements.

From these results it is concluded that dialysis against phosphate buffers between pH 7.2 - 7.8 yields optimal separations of PGB_x fractions. In general, the degree of purification attained by this dialysis procedure is minimal, since only slight changes in K_a values were found for the separated fractions as compared to the undialyzed PGB_x. Nevertheless, this dialysis method has merit since it affords an additional procedure for the separation of PGB_x into fractions according to molecular size. The advantages of this method are (1) simplicity and (2) mild conditions that assure maximum recovery of PGB_x activity.

TABLE I

The Total Weight, Molecular Weight, and In vitro Activity of PGB_x Following Dialysis

Dialysis Conditions	Fraction Weight mg	Fraction Weight % Dist.	In vitro Activity K_a	In vitro Activity % Dist.	MW
Methanol					
PGB_x Comb 2A	109.6		0.97		2077
retentate	10.8	9.5	0.84	7.5	–
dialysate	102.9	90.5	1.08	92.5	1510
Recovery	103%		113%		
pH 7.8					
PGB_x 27	143.3		1.12		2583
retentate	83.5	66.3	1.28	75.4	2804
dialysate	42.5	33.7	0.82	24.6	1849
Recovery	88%		88%		
pH 7.2					
PGB_x 27	96.2		1.12		2583
retentate	65.4	61.8	0.93	53.0	2576
dialysate	40.5	38.2	1.33	47.0	1860
Recovery	110%		106%		
pH 6.5					
PGB_x 27	132.1		1.12		2583
retentate	115.8	90.7	0.98	90.9	2694
dialysate	11.9	9.3	0.95	9.0	–
Recovery	97%		84%		

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